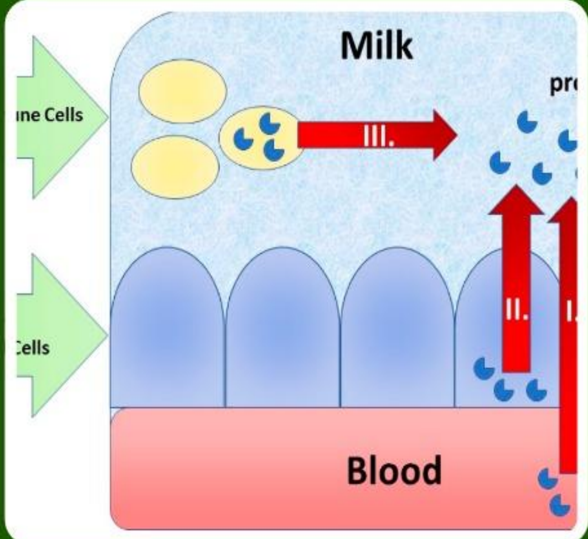
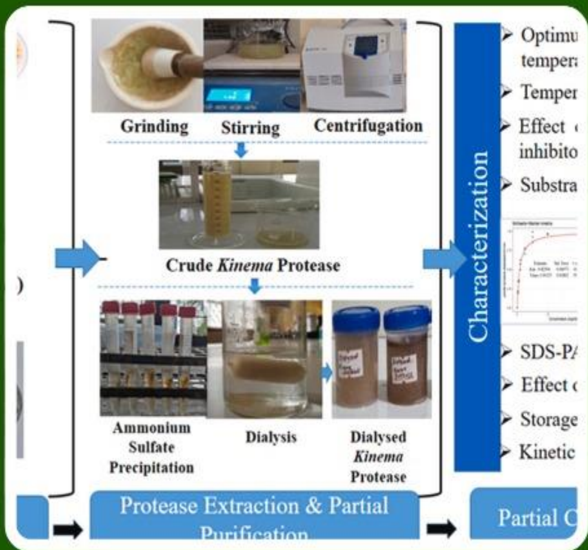
Integrating Protease Extraction from Dairy Waste and In Silico Studies for Neurodegenerative Disease Research





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Integrating Protease Extraction from Dairy Waste and In Silico Studies for Neurodegenerative Disease Research

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Abstract

Neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease are marked by the pathological accumulation of misfolded proteins, contributing to progressive neuronal damage. Proteolytic enzymes, particularly those capable of degrading β-amyloid and other neurotoxic aggregates, present a promising therapeutic avenue. However, the challenge of sustainably sourcing such enzymes limits their broader application. This study explores dairy industry waste as a viable and eco-friendly reservoir for isolating protease-producing bacteria. Following successful microbial isolation and biochemical identification, protease extracts were characterized and assessed through molecular docking simulations to evaluate their interaction with β-amyloid peptides. The results revealed significant proteolytic potential and favourable binding affinities, underscoring the enzymes' capability to disrupt amyloid aggregation. These findings highlight dairy waste as a valuable, underutilized resource for biotherapeutic enzyme discovery and lay the groundwork for future translational research into enzyme-based therapies for neurodegenerative diseases.

Keywords: Protease, dairy waste, neurodegenerative diseases, sustainability, molecular docking, biochemical assays.

1. Introduction

Proteases, also known as peptidases, are enzymes that catalyse the hydrolysis of peptide bonds, facilitating protein degradation into smaller peptides and amino acids. They play indispensable roles in various biological and industrial processes due to their ability to regulate protein turnover, modulate cellular functions, and degrade extracellular proteins [1]. Proteases are classified into six groups: serine, cysteine, aspartic, threonine, glutamic and metalloproteases based on their catalytic mechanisms, each with distinct substrate specificities and physiological functions [2].

Biologically, proteases are essential for processes such as protein digestion, immune response regulation, apoptosis, and tissue remodelling. They function as molecular chaperones, ensuring proper protein folding and degradation of misfolded or aggregated proteins that could otherwise disrupt cellular homeostasis [3]. Industrially, proteases account for approximately 60% of the global enzyme market, finding applications in food processing, pharmaceuticals, leather treatment, detergents, and waste management. Their ability to catalyse protein breakdown under diverse conditions makes them valuable in biotechnology [2].

Beyond their general enzymatic functions, proteases have been implicated in neurodegenerative diseases (NDDs), particularly Alzheimer's disease (AD). A hallmark of AD is the accumulation of β-amyloid plaques, which contribute to neuronal toxicity and cognitive decline. Certain proteases, including metalloproteases, play a crucial role in β-amyloid degradation, suggesting that enzymatic intervention could provide a potential therapeutic strategy. However, existing approaches for β-amyloid clearance, such as immunotherapies and synthetic enzyme treatments, face limitations, including high costs, limited bioavailability, and potential immune responses. This underscores the necessity for sustainable and efficient enzymatic solutions for neurodegenerative disease research.

Microbial proteases present a promising alternative due to their high catalytic efficiency, ease of production, and potential for genetic modification. Extracting proteases from sustainable sources such as dairy waste provides an eco-friendly solution while repurposing industrial byproducts. Dairy waste harbours diverse microbial communities, including bacteria capable of producing proteases with industrial and biomedical applications. Among them, *Staphylococcus aureus* is known to secrete metalloproteases that may degrade β-amyloid, offering a potential enzymatic approach for Alzheimer's research. Emerging evidence suggests that certain bacterial proteases, including those from *S. aureus*, can modulate amyloidogenic processes by cleaving β-amyloid peptides, potentially reducing plaque formation and neurotoxicity. Investigating the proteolytic potential of *S. aureus* in β-

amyloid degradation could thus provide valuable insights into alternative therapeutic strategies for Alzheimer's disease.

This study aims to investigate whether *S. aureus* from dairy waste produces metalloproteases capable of degrading β-amyloid, thereby exploring a novel and sustainable enzymatic strategy for neurodegenerative disease research. By integrating microbial enzyme extraction within silico studies, this research seeks to address current limitations in Alzheimer's therapeutics and contribute to the advancement of biotechnological solutions for disease management.

2. Methods

2.1. Sample Collection and Microbial Isolation

Soil samples were collected from a dairy processing facility in Bengaluru, Karnataka, India, to isolate potential protease-producing microorganisms. One gram of the collected soil was serially diluted in sterile 0.9% saline solution. Dilution factors of 10^{-4}, 10^{-5}, and 10^{-6} were used, and 1 mL aliquots from each dilution were plated onto nutrient agar using the spread plate method. The nutrient agar medium was prepared by dissolving 5 g of peptone, 2 g of beef extract, 5 g of NaCl, and 15 g of agar in 1000 mL of distilled water, with the final pH adjusted to 7.0. Plates were incubated at 37 °C for 24 hours. Distinct colonies were selected based on morphological characteristics and subsequently subjected to biochemical identification protocols [4].

2.2. Biochemical Identification of Bacterial Isolates

Staphylococcus aureus was identified following the guidelines outlined in *Bergey's Manual of Systematic Bacteriology* [5]. Identification was confirmed using a battery of conventional biochemical tests, including Gram staining, catalase test, methyl red test, citrate utilization test, Voges-Proskauer test, starch hydrolysis, lactose fermentation, urease test, and assays on selective media such as Mannitol Salt Agar (MSA) and Skim Milk Agar (SMA). These tests verified the species' phenotypic and enzymatic profiles [6-7].

2.3. Analytical Procedures

2.3.1. Protease Activity Assay

Protease activity was measured using a modified Lowry method. Initially, 5 mL of 0.65% casein solution was incubated at 37 °C for 5 minutes to ensure uniform mixing. Then, 0.5 mL of enzyme supernatant was added, followed by a 10-minute incubation. The reaction was terminated with 5 mL of 110 mM trichloroacetic acid. After adding an additional 0.5 mL of enzyme, the mixture was incubated at 37 °C for 30 minutes and filtered. A 2 mL aliquot of the filtrate was treated with 5 mL of 0.5 M sodium carbonate, and the reaction was stopped by adding 1 mL of 0.5 M Folin–Ciocalteu reagent. After a final 30-minute incubation at 37 °C, absorbance was recorded at 660 nm. A blank was prepared without enzyme. Enzyme activity (U/mL) was determined using a tyrosine standard curve [8-10].

2.3.2. Cell Growth Analysis

Cellular biomass was monitored by measuring the optical density (OD) of the fermentation broth at 600 nm using a UV-visible spectrophotometer. Readings were taken in 2 mL cuvettes against a sterile medium blank.

2.3.3. DPPH Antioxidant Assay

The antioxidant activity of the protease extract was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. A 0.1 mM DPPH solution in methanol was prepared. In each test, 5 mL of DPPH solution was mixed with varying volumes of the protease extract and incubated in the dark at room temperature for 30 minutes. A control was prepared by mixing 5 mL of DPPH solution with 3 mL of distilled water. Post incubation, absorbance was measured at 517 nm. Ascorbic acid served as the positive control.

2.4. Bioinformatics and Molecular Docking Studies

To investigate potential interactions between aureolysin and amyloid-beta (Aβ) peptides, we conducted protein–peptide docking simulations using the HDOCK server (http://hdock.phys.hust.edu.cn/). The crystal structure of aureolysin (retrieved from the Protein Data Bank, PDB ID: [insert if known]) was used as the receptor, and the structure of the Aβ1–42 peptide was used as the ligand. Default HDOCK parameters were applied, with global docking to predict the most favorable binding orientations. Docking poses were evaluated based on binding energy scores and the proximity of the Aβ peptide to the catalytic site of aureolysin.

Additionally, substrate specificity data for aureolysin were obtained from the MEROPS peptidase database (family M04.009) to assess cleavage site compatibility between aureolysin and Aβ peptide sequences.

3. Results

3.1. Microbial Identification and Characterization

The isolate obtained from dairy waste soil samples exhibited distinct morphological and biochemical characteristics indicative of *Staphylococcus aureus*. Gram staining revealed Gram-positive cocci arranged in grape-like clusters (Figure 1). Biochemical profiling further supported the identification, as the isolate tested positive for catalase, Voges-Proskauer, starch hydrolysis, lactose fermentation, citrate utilization, and protease production on skim milk agar (Figure 2). Negative results were observed for the methyl red and urease tests. Growth on Mannitol Salt Agar was accompanied by a yellow colour change, confirming mannitol fermentation, a hallmark of *S. aureus*.

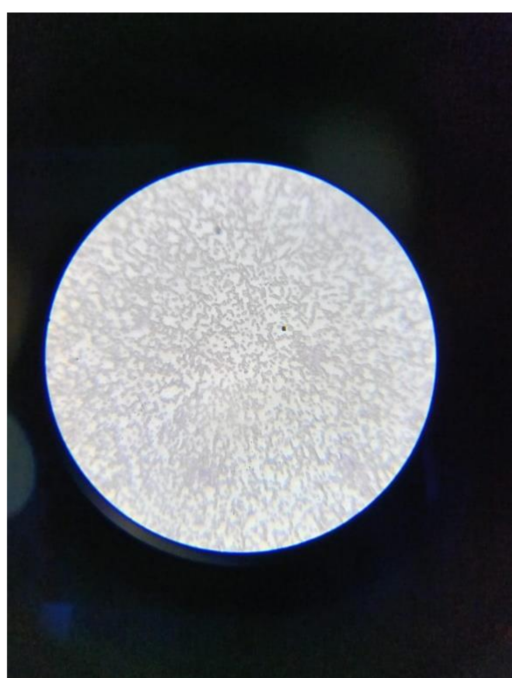

Figure 1. Gram staining of the isolated bacterial strain showing characteristic Gram-positive cocci arranged in clusters, indicative of *Staphylococcus aureus.* The violet coloration confirms retention of crystal violet stain, a hallmark of Gram-positive cell walls.

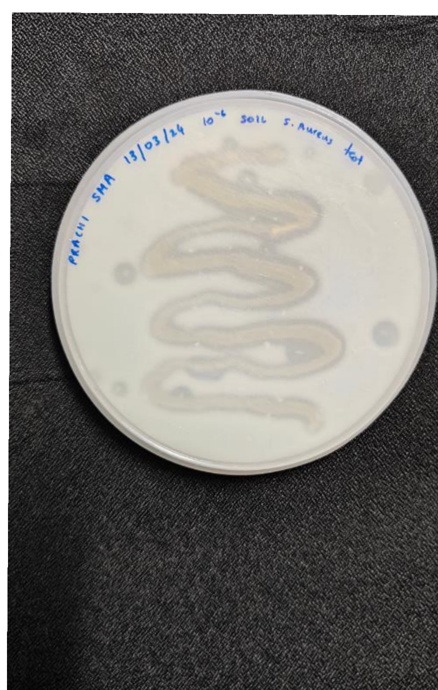

Figure 2. Positive result of the skim milk agar test indicating protease activity by the isolated bacterial strain. Clear zones of casein hydrolysis around the bacterial streaks confirm extracellular protease secretion, demonstrating the organism's ability to degrade milk proteins.

Table 1. Summary of biochemical tests performed for the identification of the bacterial isolate. The results support the identification of the organism as Staphylococcus aureus, based on its enzymatic activities and metabolic characteristics. Positive outcomes in catalase, VP, starch hydrolysis, and citrate utilization tests, along with growth on selective media such as Mannitol Salt Agar and Skim Milk Agar, confirm its proteolytic and fermentative capabilities.

Test	**Observation**	**Inference**
Catalase Test	Positive	Presence of catalase enzyme
Methyl Red Test	Negative	No stable acid production
Voges-Proskauer Test	Positive	Acetoin production confirmed
Starch Hydrolysis	Positive	Amylase activity
Lactose Fermentation	Positive	Acid production from lactose
Mannitol Salt Agar (selective media)	Growth, yellow colour	Fermentation of mannitol
Skim Milk Agar (selective media)	Positive	Protease activity (casein hydrolysis)
Urease Test	Negative	No urease production
Citrate Utilization	Positive	Ability to utilize citrate

3.2. Protease Activity Assay

Protease production was quantitatively assessed using a modified Lowry method. Casein (0.65%) served as the substrate, and the reaction was terminated with trichloroacetic acid. The digested peptides were then measured with the Folin–Ciocalteu reagent. The control (enzyme-free blank) exhibited an absorbance of 0.004, while the sample containing the enzyme showed a significant increase to 0.082. This corresponds to a 20.5-fold elevation in absorbance, indicative of substantial proteolytic activity.

3.3. Cell Growth Analysis

Growth kinetics of the isolated strain were assessed by measuring optical density (OD) at 600 nm. The blank (uninoculated medium) registered an OD of 0.004, while the fermentation broth containing *S. aureus* cells reached an OD of 0.693. This indicates robust cell proliferation under the tested conditions.

3.4. Antioxidant Potential of Protease Extract

The antioxidant activity of the protease extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. A concentration-dependent decline in absorbance at 517 nm was observed, signifying effective free radical scavenging by the protease extract (Figure 3). These results suggest the potential of the enzyme to mitigate oxidative stress, a contributing factor in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease.

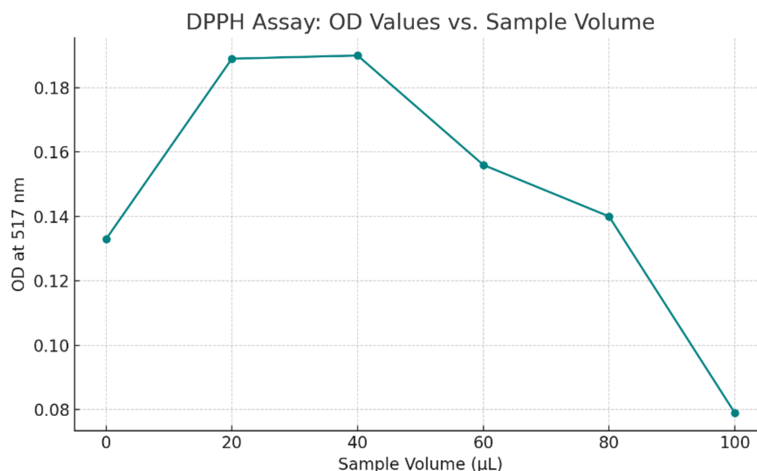

Figure 3: Protease extracted from microbial culture exhibiting antioxidant potential against DPPH.

3.5. In Silico Bioinformatics and Molecular Docking Analysis

Protein–peptide docking simulations performed with HDOCK predicted strong interactions between Staphylococcus aureus aureolysin (PDB ID: 1BQB) and amyloid-beta 1–42 peptide (PDB ID: 5BUO). The four highest-ranked docking models as shown in Figure 4 exhibited binding scores of –223.00, –219.43, –217.98, and –216.90, respectively, indicating highly favorable binding energies. Corresponding confidence scores were 0.8115, 0.8004, 0.7957, and 0.7922, supporting the reliability of these predicted complexes.

In all top models, the Aβ peptide was oriented with potential cleavage sites in proximity to the catalytic zinc-binding site of aureolysin. Notably, hydrophobic regions of Aβ, including the Leu17–Val18 segment, were positioned within the catalytic cleft, consistent with aureolysin's known preference for hydrophobic residues at P1 and P1' cleavage positions, as reported in MEROPS (family M04.009).

Interface analysis revealed specific contact residues on both the receptor and ligand sides, highlighting critical interaction points that stabilize the complex. These receptor–ligand interface residues further support the feasibility of substrate recognition and processing by aureolysin.

Collectively, these results suggest a strong structural basis for potential enzymatic degradation of Aβ by aureolysin and provide a rationale for further biochemical validation.

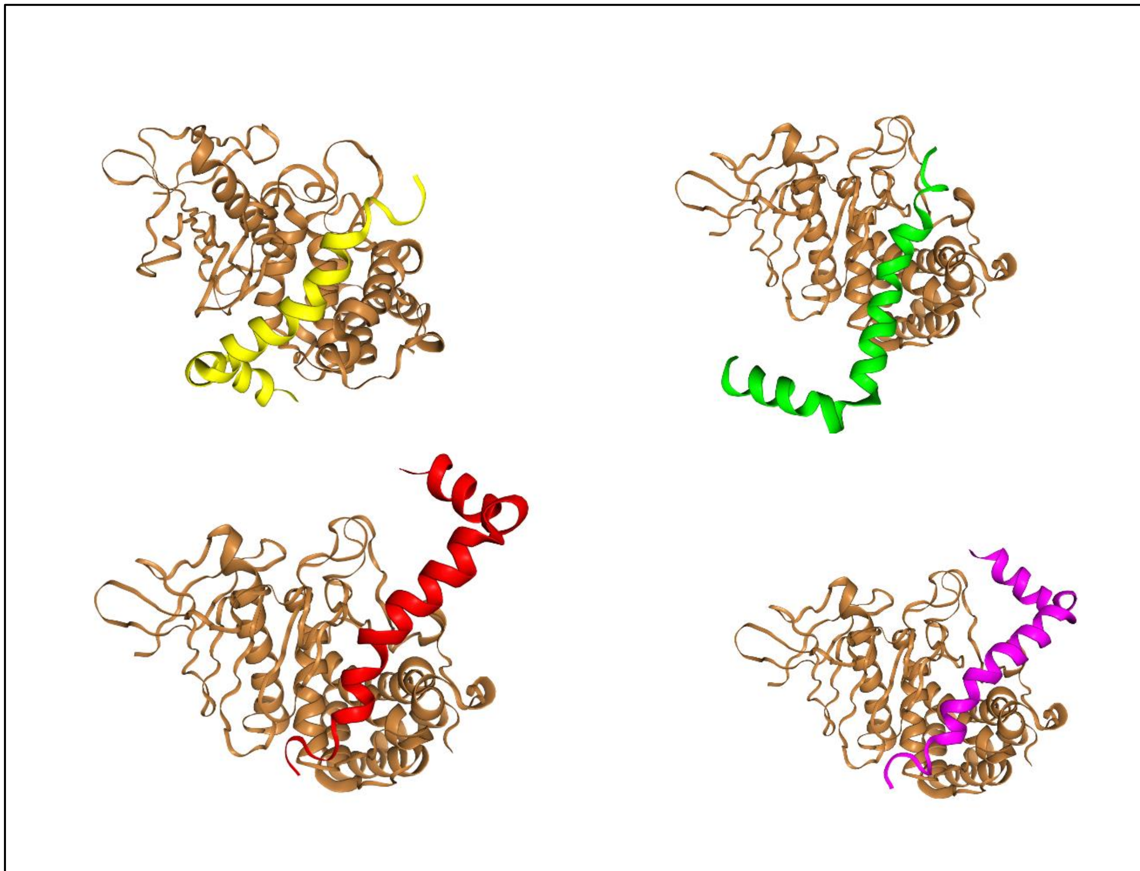

Figure 4 : Top four docking simulations of aureolysin (brown) with amyloid beta. Each model is represented in a different colour: yellow, green, red, and pink. The corresponding binding scores for the top-ranked docking models are –223.00 (yellow), –219.43 (green), –217.98 (red), and –216.90 (pink), respectively. These models were created using HDOCK and illustrate the most favourable binding conformations

4. Bioinformatics Analysis

The bioinformatics analysis yielded valuable insights into the interaction profiles of the selected enzymes with β-amyloid peptides.

Structural visualization using Chimera and Avogadro allowed for detailed examination of active sites and conformational features critical for binding. Energy minimization in Swiss-PDB Viewer improved the overall stability of the protein models, as evidenced by reduced steric clashes and improved Ramachandran plot statistics.

Overall, the results support the hypothesis that specific enzymes may possess therapeutic potential in modulating β-amyloid aggregation, paving the way for further in vitro and in vivo validation studies.

5. Discussion

The biochemical profile of the isolated strain confirmed the presence of Staphylococcus aureus, a bacterium well known for its production of metalloproteases. Positive results observed on skim milk agar plates supported the strain's proteolytic capabilities, with clear zones of casein hydrolysis suggesting active secretion of proteases. Quantitative enzyme assays further validated these findings, demonstrating significant casein degradation. Given the structural complexity and hydrophobic nature of casein, its breakdown serves as a functional analog to β-amyloid degradation potential. Similar studies were also observed in which clinical isolates of *Staphylococcus aureus* obtained from atopic dermatitis patients exhibited pronounced proteolytic activity, as evidenced by the formation of distinct clearance zones on skim milk agar plates, indicative of extracellular caseinolytic enzyme secretion. Quantitative assessment using azocasein hydrolysis and chromogenic synthetic substrates further validated the presence of active metalloproteases and serine proteases. The coordinated activity of these

proteolytic enzymes not only underscores the pathogenic adaptability of *S. aureus* in cutaneous environments but also highlights their potential relevance as functional analogs in the degradation of structurally complex, hydrophobic substrates such as β-amyloid aggregates. This enzymatic profile suggests a mechanistic parallel that may be exploited in the context of amyloidogenic protein clearance studies [11].

In addition to proteolytic activity, the protease extract exhibited notable antioxidant properties, as evidenced by the DPPH assay. A clear dose-dependent decrease in optical density values was observed with increasing sample volume (20 µL to 100 µL), highlighting the extract's capacity to neutralize free radicals. The dual functionality of proteolysis and antioxidative stress mitigation presents an attractive therapeutic profile, especially for diseases like Alzheimer's, where oxidative damage and protein aggregation coexist. Studies by de Araújo et al. [12] provide compelling evidence supporting the antioxidant potential of protease extracts derived from microbial and plant sources, respectively. Sahu et al. isolated a thermostable protease from *Bacillus cereus* and demonstrated its significant antioxidant activity using DPPH and ABTS radical scavenging assays. The extract showed a clear concentration-dependent free radical neutralization, indicating its strong potential as a dual-function enzyme with both proteolytic and antioxidative capacities. Similarly, de Araújo et al. investigated enzymatic extracts from pineapple waste and observed notable antioxidant activity through DPPH assays, revealing that even agro-industrial byproducts can serve as rich sources of bioactive compounds. These findings collectively suggest that proteases, beyond their classical role in protein hydrolysis, can contribute to oxidative stress mitigation. This dual functionality enhances their therapeutic relevance, particularly in chronic diseases like Alzheimer's, where oxidative imbalance and protein aggregation are central pathological features.

Based on the biochemical properties of staphylococcal proteases, specifically aureolysin, as described by Stach et al. [13], we used in silico methods to investigate the possibility of its interaction with amyloid-beta (Aβ) peptides, which are a defining feature of the pathophysiology of Alzheimer's disease. The top four models showed docking scores of –223.00, –219.43, –217.98, and –216.90, with confidence scores ranging from 0.8115 to 0.7922. Protein–peptide docking using the HDOCK server revealed strong binding affinities between aureolysin (PDB ID: 1BQB) and Aβ1–42 (PDB ID: 5BUO). Interestingly, the hydrophobic section of Aβ (Leu17–Val18) was consistently positioned near the catalytic zinc-binding site of aureolysin by the docking models. This arrangement is in line with substrate specificity insights from the MEROPS database (M04.009 family), which shows that aureolysin would rather to cleave in aliphatic or hydrophobic.

Importantly, no physiological substrates for aureolysin within human tissues have been reported, reducing concerns regarding unintended proteolytic activity and increasing its attractiveness as a candidate for therapeutic development. The integration of biochemical, enzymatic, antioxidant, and computational analyses suggests that S. aureus-derived aureolysin holds potential as a novel biocatalyst capable of targeting and degrading amyloid aggregates in neurodegenerative contexts.

Although our findings present compelling preliminary evidence, further experimental validation remains essential. Future directions will focus on in vitro cleavage assays using synthetic Aβ peptides, kinetic profiling to assess catalytic efficiency, and specificity studies to ensure selectivity toward pathological aggregates without off-target degradation. Additionally, structure-based engineering could be pursued to optimize aureolysin variants for enhanced efficacy and therapeutic compatibility.

Overall, this study proposes a sustainable, microbial-based approach to amyloid clearance, opening new avenues for enzyme therapy development against Alzheimer's disease.

6. Conclusion

This study demonstrated that Staphylococcus aureus isolated from dairy waste is a potent source of proteases, including potential metalloproteases capable of protein degradation. With confirmed enzymatic activity and promising biochemical traits, these findings pave the way for further in silico analysis and therapeutic exploration in Alzheimer's research. The project supports the concept of sustainable biotechnology by leveraging waste-derived bioresources for high-value medical research.

7. Acknowledgement

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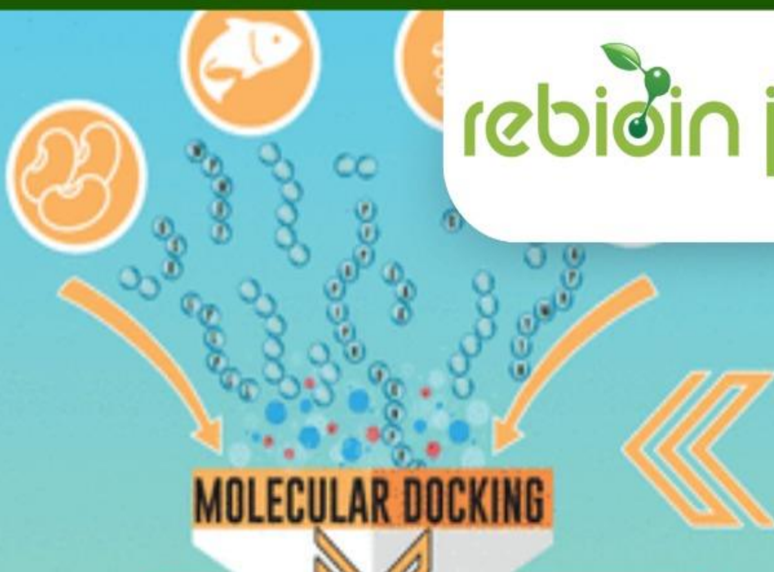

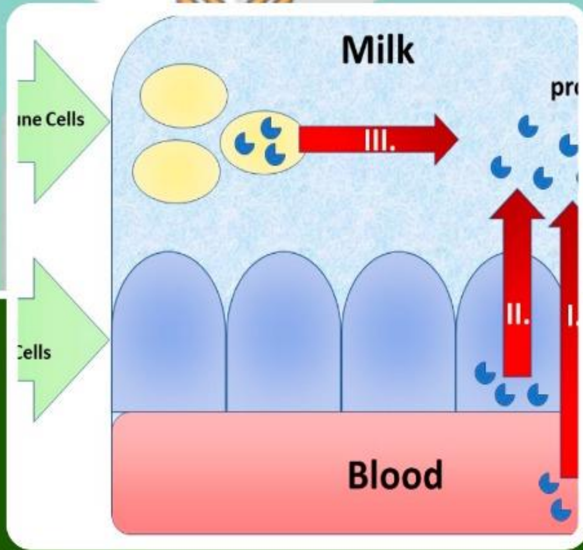

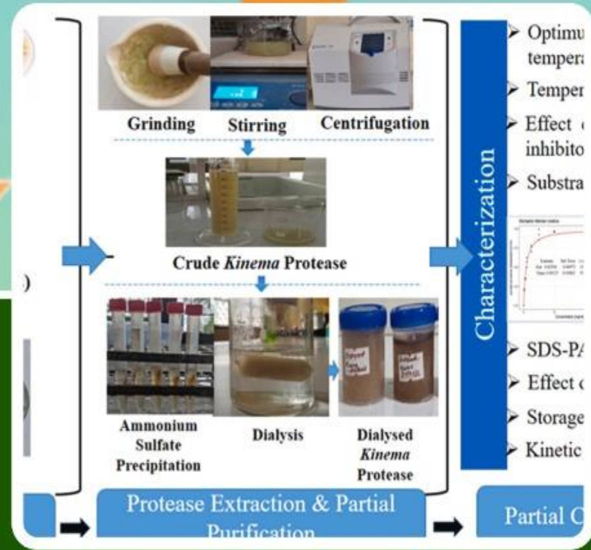

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